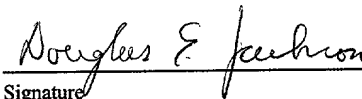


FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		Attorney's Docket No.:
<b>TRANSMITTAL LETTER TO THE UNITED STATES</b> <b>DESIGNATED/ELECTED OFFICE (DO/EO/US)</b> <b>CONCERNING A FILING UNDER 35 U.S.C. 371</b>		<b>XI/P6155US0</b>
		U.S. Appln. No.:
INTERNATIONAL APPLICATION NO. PCT/FR97/00897	INTERNATIONAL FILING DATE 22 MAY 1997	PRIORITY DATE CLAIMED 23 MAY 1996
Title of Invention IMMORTAL AVIAN CELLS		<b>09/194025</b>
Applicant(s) for DO/EO/US BOUQUET, Jean-Francois et al.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SEBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19<sup>th</sup> month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A <b>copy</b> of the International Application as filed (35 U.S.C. 371 (c)(2)) <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input checked="" type="checkbox"/> A <b>translation</b> of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)) <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments had NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input type="checkbox"/> An <b>oath</b> or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>		
<b>Items 11. To 16. Below concern document(s) or information included:</b> <ol style="list-style-type: none"> <li>11. <input type="checkbox"/> An <b>Information Disclosure Statement</b> under 37 C.F.R. 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An <b>Assignment</b> document for recording. A separate cover sheet in compliance with 37 C.F.R. 2.28 and 3.31 is included.</li> <li>13. <input checked="" type="checkbox"/> A <b>First preliminary amendment</b>.  <input type="checkbox"/> A Second or subsequent preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>16. <input type="checkbox"/> Other:  <input type="checkbox"/> Small Entity Statement  <input type="checkbox"/>  <input type="checkbox"/> </li> <li><input type="checkbox"/> A copy of the Notification of Missing Requirements under 35 U.S.C. 371.</li> <li><input type="checkbox"/> In the event that a petition for extension of time is required to be submitted herewith, and in the event that a separate petition does not accompany this response, applicant hereby petitions under 37 CFR 1.136(a) for an extension of time of as many months as are required to render this submission timely. Any fee is authorized in 17(c).</li> </ol>		
Date: 19 November 1998		

U.S. Application No. (if known, see 37 CFR 1.5)		International Application No. PCT/FR97/00897		Attorney's Docket No.: XI/P6155USO	
17. <input checked="" type="checkbox"/> The following fees are submitted:				Calculations - PTO use only	
<b>Basic National Fee</b> (37 CFR 1.492 (a) (1)-(5);					
<input checked="" type="checkbox"/> Search Report has been prepared by the EPO or JPO ..... \$ 840.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO ..... \$ 670.00					
<input type="checkbox"/> No Int'l Prelim. Exam. fee paid to USPTO but Int'l Search fee paid to USPTO ..... \$ 760.00					
<input type="checkbox"/> Neither Int'l Prelim. Exam. fee nor Int'l Search fee paid to USPTO ..... \$ 970.00					
<input type="checkbox"/> Int'l Prelim. Exam. fee paid to USPTO & all claims satisfied PCT Art. 33(2)-(4) ..... \$ 96.00					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>\$ 840.00</b>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 mos. <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number filed	Number extra	Rate		
Total claims	13 - 20 =		X \$ 18.00	\$	
Independent claims	04 - 03 =		X \$ 78.00	\$	
Multiple Dependent Claim(s) (if applicable)			+ \$260.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$ 840.00</b>	
Reduction of 1/2 for filing by small entity, if applicable. Small Entity Statement must also be filed.				\$	
<b>SUBTOTAL =</b>				<b>\$ 840.00</b>	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 mos. <input type="checkbox"/> 30 mos. From the earliest claimed priority date (37 CFR 1.492(f)).				+ \$	
<b>TOTAL NATIONAL FEE =</b>				<b>\$ 840.00</b>	
Fee for recording the enclosed assignment, accompanied by a cover sheet \$40.00 per property				+ \$	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$ 840.00</b>	
				Amount to be:	\$
				Refunded	
				Charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>840.00</u> to cover the above fees is enclosed.					
b. <input type="checkbox"/> Please charge my Deposit Account No. 12-0555 in the amount of \$ _____ to cover the above fees.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees required or credit overpayment to Deposit Account No. 12-0555.					
<b>Note:</b> Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be be filed and granted to restore the application to pending status.					
Please address all correspondence to <u>THOMAS P. SARRO</u> at the address (below) of <u>Customer Number: 000881</u>					
SEND ALL CORRESPONDENCE TO:					
Larson & Taylor Transpotomac Plaza 1199 North Fairfax Street, Suite 900 Alexandria, Virginia 22314-1437			 Signature Name: Douglas E. Jackson Registration: 28518		
Date: <u>19</u> November 1998					

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Patent

In re application of **BOUQUET, et al.** )  
New Application ) Atty's Dckt: XI/P6155US0  
Filed: On even date herewith ) Application Branch  
For: IMMORTAL AVIAN CELLS )

**PRELIMINARY AMENDMENT**

Hon. Assistant Commissioner of Patents  
Washington, D.C. 20231

S I R:

Preliminary to the examination thereof, please amend the above-identified application as follows:

**IN THE CLAIMS:**

Claim 5, line 2, delete "one of Claims 1 to 4", and insert therefor --claim 4--.  
Claim 9, line 1, delete "any one of Claims 5 to 8", and insert therefor --claim 5--.  
Claim 10, line 1, delete "any one of Claims 5 to 9", and insert therefor --claim 5--.  
Claim 11, line 1, delete "any one of Claim 5 to 10", and insert therefor --claim 5--.  
Claim 12, line 1, delete "any one of claims 5 to 11", and insert therefor --claim 5--.  
Claim 13, line 2, delete "any one of Claim 5 to 12", and insert therefor --claim 5--.

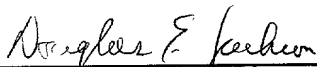
**REMARKS**

The above amendments are being made in order to place the application in better condition for examination and to reduce the filing fee.

Favorable consideration is respectfully requested.

Respectfully submitted,

Date: November 18, 1998

  
By: Douglas E. Jackson  
Registration No. 28518

**LARSON & TAYLOR**

1199 North Fairfax Street, Ste. 900 • Alexandria, Virginia 22314 • (703) 739-4900

IMMORTAL AVIAN CELLS

The present invention relates to avian cell lines and their derivatives.

5 It is not possible to establish cell lines spontaneously from organs taken from avian species, as can be done in the case of some organs derived from mammalian species.

10 The only available cell lines to date were obtained using the transforming properties of certain avian viruses which possess oncogenic properties, such as the retroviruses of the avian leukosis group or Marek's disease virus, or certain chemical molecules such as methylcholanthrene and diethylnitrosamine.

15 For the most part, these cell lines are considerably transformed, which renders them unsuitable for multiplying vaccine viruses.

20 Authors are adopting a novel approach which consists in introducing into cells a vector which does not exhibit any oncogenic character but which is able to integrate, into these cells, a gene which is selected for its capacity to induce immortalization.

25 The first tests were carried out using vectors which integrate avian retrovirus genes such as erbA and erbB.

30 French Patent Application FR-A-2 596 770 proposes an immortalization method in which a culture of avian or mammalian cells is infected with a vector or a system which, while not being oncogenic for the said cells, is able to integrate a gene selected from v-myb, v-ets and v-erbA into these cells. The AMV, E26 and XJ12 viruses, with this latter being a virus derivative of the AEV virus in which the oncogenic v-erbB gene has been deleted, can be appropriate vectors.

35 In practice, while these tests made it possible to obtain established cell lines from cells of the haematopoietic cell line, they did not give the expected results in the case of chick embryo cells in adherent culture, such as fibroblasts or epithelial cells.

It was possible to obtain untransformed avian cell lines of the myeloblastoid type (blood cells) using the oncogene myb (International Patent Application WO91/18971).

5 In parallel, authors have proposed using the early t and T genes of the simian virus SV40 for immortalizing cells derived from different mammalian tissues (D.S. Neufeld et al., Molecular and Cellular Biology, August 1987, 2794-2802, O. Kellermann and F. Kelly,  
10 Differentiation 1986, 32 : 74-81 and French Patent Application FR-A-2 649 721).

For its part, French Patent Application FR-A-2 649 721 proposes a method of conditional immortalization which, it is claimed, can be used for any cell type and  
15 in any species, with the aim in this case being that of remedying the drawback of the high degree of specificity of the conventional approaches (limitation to particular species and/or to particular cell types): transformation of cells with a transforming virus (adenovirus, Epstein-  
20 Barr virus, certain papovaviruses such as the SV40 virus or polyoma virus; for example, the SV40 virus is indicated as only transforming rodent cells and human cells); transfection with constructs which contain a transforming gene which is linked to a viral promoter;  
25 transfection with a transforming gene which is linked to a cellular promoter. The choice of this patent application falls on a construct which combines a DNA fragment from the regulatory sequence of vimentin and a DNA fragment which encodes an immortalizing gene, which  
30 construct can be the T antigen of the SV40 virus under the control of the inducible promoter of vimentin. This document never mentions the avian species.

The actual use of such viral oncogenes has never been described in the avian species, apart from the use  
35 of the 12S form of the E1A protein of human adenovirus 5, which made it possible to immortalize quail epithelial cells (Guilhot et al. (1993), Oncogene 8 : 619-624).

Contrary to all expectations, the inventors succeeded in producing immortal, untransformed avian cell lines.

More generally, the inventors have found that it  
5 was possible to prepare immortal, untransformed avian cell lines which are resistant to apoptosis even from cells of avian tissues, that is to say from cells other than circulating blood cells or haematopoietic cells.

The present invention therefore relates to  
10 immortal, untransformed avian cells which are resistant to apoptosis and which derive, in particular, from avian tissues, that is to say from cells other than blood cells or haematopoietic cells, in particular fibroblasts and epithelial cells, for example from embryos.

15 The present invention more especially relates to an untransformed, immortal avian cell line which is selected from the group consisting of:

- cell line TDF-2A bcl-2, which is deposited in the CNCM (Collection Nationale de Cultures de Micro-organismes de l'Institut Pasteur [Pasteur Institute National Collection of Microorganism Cultures]) under  
20 reference number I-1709
- cell line TCF-4.10, which is deposited in the CNCM under reference number I-1710
- 25 - cell line TCF-4.10 bcl-2, which is deposited in the CNCM under reference number I-1711

bcl-2 denotes that the cells of the cell line functionally integrate the bcl-2 gene, which confers on them resistance to apoptosis (WO-A-93/20200, which is  
30 hereby incorporated by reference).

The invention naturally covers the cells which are derived from these cell lines. By this, it is to be understood that it is not only the cells as deposited in the CNCM under the indicated references which are  
35 covered but also the cells which constitute the progeny of these deposited cells, i.e., on the one hand, those which are obtained by simple multiplication and which may undergo mutations during these multiplications and, on the other hand, those which are obtained after

deliberate modification, which are then termed the derived cells, and, of course, also those which have undergone the two types of modification.

The invention therefore also covers the derived  
5 cells which are obtained by modifications of the above cells. These modifications may consist in:

- Inserting one or more expression cassettes, each of which comprises one or more nucleotide sequences encoding a molecule of industrial relevance, with these  
10 expression cassettes being able to produce this molecule following insertion into the cells of the invention. The skilled person is fully conversant with the technique. Molecules of industrial relevance which may be mentioned, in particular, are viral subunits of the  
15 peptide, protein or glycoprotein type, in particular for use in a vaccine or a diagnostic reagent, protein molecules such as hormones, etc.

- Chronically infecting with a virus which is able to multiply in these cells, for virus or vaccine production  
20 purposes, with or without prior modification of the sensitivity towards this virus. The infection may also not be chronic but carried out on a batch of cells which is selected for the viral multiplication.

(The modifications described below are to be understood  
25 as preferably and advantageously being combined with the preceding two types of modification).

- Introducing survival or anti-apoptotic genes other than bcl-2, such as the genes which encode the human adenovirus p19E1B (Rao et al. (1992), Proc. Natl. Acad.  
30 Sci. USA 89 : 7742-7746), the Epstein Barr virus LMP-1 (Gregory et al. (1991), Nature 349 : 612-614) and BHRF1 (Pearson et al. (1987), Virology 160 : 151-161), the herpes simplex virus ICP34.5 (Chou and Roizman (1992), Proc. Natl. Acad. Sci. USA 89 : 3266-3270) and the  
35 baculovirus p35 (Clem et al. (1991), Science 254 : 1388-1390) proteins in order to render these cell lines more resistant to the culture conditions, in particular for maintaining confluence.

- Overexpressing genes which are involved in controlling the cell cycle using vectors which are suitable for increasing the rate of proliferation. Thus, it has been demonstrated that, in certain cases, overexpressing cyclin-encoding genes leads to the cell cycle being shortened and therefore to the rate of proliferation being increased (Rosenberg et al. (1995), *Oncogene* 10 : 1501-1509; Quelle et al. (1993), *Genes and Dev.* 7 : 1559-1571).
- Modifying the viral sensitivity spectrum of the cell lines by integrating genes which encode receptors for the viruses of interest, with a view to multiplying these viruses.
- Reference may be made to the mammalian species, where expression of the receptor for the measles virus (CD46) by murine cells, which are normally non-permissive for the virus, results in these cells becoming sensitive to this virus and being able to replicate it (Naniche et al. (1993), *J. Virol.* 67 : 6025-6032). The interest is, in particular, in rendering cells sensitive to a virus in order to produce the virus on these cells.
- Integrating oncogenes which are able to accelerate cell growth.

It is self-evident that the derived cells according to the invention may comprise one or more of the above-described modifications.

The invention also relates to a method for producing molecules of industrial relevance or viruses, which method comprises culturing the above-described cells.

The present invention is directed, in particular, towards producing molecules or viruses for creating diagnostic reagents or vaccines, or else towards producing molecules of therapeutic relevance.

The invention will now be described in more detail with the aid of embodiments which are taken by way of non-limiting examples and with reference to the attached drawing, in which:



- Figure 1 shows the structure of the vector pDAMT, which is used to prepare the cell line TDF-2A, and in which:

LTR: direct repeat sequence (long terminal repeat)

5 LTR: deleted LTR

MTI: murine metallothionein I promoter

SV40 T+t: SV40 early region

SV40: SV40 promoter

- Figure 2 shows the structure of the vector ppHMT, which is used to prepare the cell line TCF-4.10, and in which:

LTR: direct repeat sequence (long terminal repeat)

phleo: gene for resistance to phleomycin

SV40pA: SV40 polyA

15 MTI: murine metallothionein I promoter

SV40 T+t: SV40 early region

#### **EXAMPLE 1 = Production of the TDF-2A cell line**

#### 20 **I. Description of its origin and its characteristics**

##### **1.1 Description of the vector employed : vector pDAMT**

It comprises the SV40 virus early region  
25 (encodes the T and t antigens) (HindIII/BamHI fragment) (Fiers et al. (1978), Nature 273 : 113-120) under the control of the mouse metallothionein I promoter (EcoRI/BglII fragment with the BglII site being transformed into a HindIII site) (Durnam et al. (1980),  
30 Proc. Natl. Acad. Sci. USA 77 : 6511-6515 ; Brinster et al. (1982), Nature 296 : 39-42).

The EcoRI/EcoRI fragment containing this transcription unit, derived from the vector pMTSVneo (Peden et al. (1989), Exp. Cell. Res. 185 : 60-72), was  
35 inserted into the XbaI site of the vector pDA1 (Aubert et al. (1991), J. Cell. Biol. 113 : 497-506). This latter vector is essentially derived from the genome of the Rous sarcoma-associated virus 2 (RAV-2) following modification of the 3' LTR. Thus, the U3 region of the

RAV-2 3' LTR was deleted and linked to the R and U5 regions isolated from the Rous sarcoma-associated virus 1 (RAV-1) LTR. The vector also carries a transcription unit which contains the gene for resistance to neomycin under the control of the SV40 promoter derived from the vector pSV2neo (Southern and Berg (1982), J. Mol. Appl. Genet. 1 : 327-341). See Figure 1.

**1.2.** Establishment of the cell line and demonstration that it is immortalized.

Cells derived from 14-day Muscovy duck embryos were transfected with vector pDAMT using the dimethyl sulphoxide (DMSO) method described by Kawai and Nishizawa (1984), Mol. Cell. Biol. 4 : 1172-1174. The transfected cells are then selected by applying geneticin G418 (150 µg/ml) for 15 days. The resistant clones are then subcultured regularly at a rate of from 1 to 2 passages per week. After this 3-month period of active proliferation, the cells entered into a crisis period during which most of the cells died. After this period, which lasted approximately 2 months, several clones resumed active proliferation, suggesting that they had been immortalized.

The TDF-2A cell line is thus derived from 2 cultures.

It was studied in more depth.

The TDF-2A cells achieved 200 passages, that is approximately 460 generations, and were thus maintained continuously in culture for more than 600 days. By comparison, control cells, which are not expressing the SV40 virus early region, cannot be maintained in culture for more than 20 passages.

**1.3.** Proliferation characteristics.

The immortalized cells are cultured at 38°C, in a roller bottle, in a medium containing 6% 10 × HAM F-10, 4% 10 × 199 HANKS, from 2.95% to 4% tryptose broth phosphate, from 5.6% to 2.5% sodium bicarbonate, 0.1% 100 × vitamin BME, 3% foetal calf serum, from 5% to 1% kanamycin and from 0.5% to 1% vancomycin.

Under these conditions, their rate of doubling is once every 24 hours.

#### 1.4. Expression of the T antigen.

It was verified, by means of indirect immunofluorescence or indirect immunophosphatase using an antibody which is specific for the T antigen (Pab 101 : Santa Cruz Biotechnology ref. sc147), that all the cells express the T antigen in their nucleus, indicating that they have all integrated the vector.

This integration was additionally demonstrated by means of Southern blotting. The genomic DNA of the immortalized fibroblasts was digested with the restriction enzymes XbaI and BstXI. Hybridization with a probe which was specific for the T antigen (1018 bp NdeI/NdeI fragment) verified that the transcription unit, which expressed the immortalizing gene and which was inserted into the TDF-2A cells, had not undergone any major rearrangements. This was indicated by the fact that the sizes of the hybridization fragments obtained were in accordance with the expected sizes.

#### 1.5. Absence of tumorigenic capacity.

The immortalized cells do not exhibit any tumorigenic capacity. They are incapable of forming colonies in semi-solid medium or of forming tumours on hen or duck egg chorioallantoic membrane. They are also incapable of forming tumours on nude mice, and on one-day old SPF (pathogen-free) ducklings and chicks.

#### 1.6. Karyotype.

The karyotype of the TDF-2A cells was studied at the 114th and 135th passages. This verified that the cells were indeed of avian origin, with the microchromosomes characteristic of this species being present. Furthermore, the chromosomes which were observed are representative of the chromosomes which are encountered in primary duck embryo cells, thereby confirming the origin of the cell line.

### II. Properties.

The TDF-2A cells exhibit, in particular, a sensitivity to the duck-specific viruses, such as

adenovirus, parvovirus and reovirus, which are customarily replicated on primary duck embryo cells. These viruses can therefore be produced on this cell line.

5 **EXAMPLE 2 : Characterization of the TDF-2A cell line by identifying the integration sites.**

The genomic DNA of the TDF-2A cells, which was prepared from cells derived from the 114th and 135th passages, was digested with the restriction enzymes  
10 BglII and KpnI. The DNA, which had been treated in this way, was then subjected to gel electrophoresis, followed by transfer to a nylon membrane; it was then hybridized with a probe which was specific for the T antigen (1018 bp NdeI/NdeI fragment). For example, digestion with  
15 BglII gives rise to two hybridization bands of large size (approximately 15 and 23 kb), suggesting the existence of two integration sites. Digestion with KpnI gives rise to one major band of large size (approximately 20 kb) and to at least one minor band,  
20 thereby confirming the existence of at least two integration sites.

**EXAMPLE 3 : Production of the TCF-4.10 cell line**

1. Description of its origin and its characteristics  
25

1.1. Description of the vector employed: vector pphMT

It comprises the SV40 virus early region (encodes the T and t antigens) (HindIII/BamHI fragment)  
30 (Fiers et al. (1978), Nature 273: 113-120) under the control of the mouse metallothionein I promoter (EcoRI/BglII fragment with the BglII site being transformed into a HindIII site) (Durnam et al. (1980), Proc. Natl. Acad. Sci. USA 77: 6511-6515; Brinster  
35 et al. (1982), Nature 296: 39-42).

The EcoRI/EcoRI fragment containing this transcription unit, derived from the vector pMTSVneo (Peden et al. (1989), Exp. Cell. Res. 185: 60-72), was inserted into the EcoRI site of the vector pUT507

(marketed by CAYLA-FRANCE), being located 3' of the region for expressing the gene for resistance to phleomycin (Figure 2). The structure of vector pUT507 is described in Mulsant et al. (1988), Somatic Cell and  
5 Molecular genetics 14: 243-252.

**1.2.** Establishment of the cell line and demonstration that it is immortalized.

Fibroblasts derived from chick embryos were transfected with vector pphMT using the dimethyl  
10 sulphoxide (DMSO) method described by Kawai and Nishizawa (1984), Mol. Cell. Biol. 4: 1172-1174. The transfected cells are then selected by progressively applying (from 10 µg/ml to 50 µg/ml) phleomycin for 15 days. The resistant clones are then subcultured  
15 regularly at a rate of from 1 to 2 passages per week. After a period of active proliferation of approximately 2 months, the cells entered into a crisis period when cell growth is very weak and during which there is a very high rate of mortality. After a period which  
20 lasted from 3 to 4 months, a few cells of the TCF-4.10 clone resumed active proliferation, suggesting that they had been immortalized.

The TCF-4.10 cells thus achieved 200 passages in culture, that is approximately 400 generations, and  
25 were maintained in culture for 3 years. By comparison, control fibroblasts, which are not expressing the SV40 virus early region, cannot be maintained in culture for more than 20 to 30 passages.

**1.3.** Proliferation characteristics.

30 The immortalized fibroblasts are cultured at 38°C in a medium containing 6% 10 × HAM F-10, 4% 10 × 199 HANKS, from 2.95% to 4% tryptose broth phosphate, from 5.6% to 2.5% sodium bicarbonate, 0.1% 100 × vitamin BME, 3% foetal calf serum, from 5% to 1%  
35 kanamycin and from 0.5% to 1% vancomycin. Under these conditions, their rate of doubling is 0.7 times per 24 hours.

## 2.2. Expression of the T antigen.

It was verified, by means of indirect immuno-  
fluorescence or indirect immunophosphatase using an  
antibody which is specific for the T antigen (Pab 101:  
5 Santa Cruz Biotechnology ref. sc147), that all the  
cells express the T antigen in their nucleus, indicat-  
ing that they have all integrated the vector.

## 2.3. Absence of tumorigenic capacity

The immortalized fibroblasts do not exhibit any  
10 tumorigenic capacity. They are incapable of forming  
tumours on hen or duck egg chorioallantoic membrane.

## 3. Properties.

The TCF-4.10 cells exhibit, in particular, a  
sensitivity to avian viruses. Viruses which may, in  
15 particular, be mentioned are the avian poxviruses, such  
as canarypox or fowlpox, or else the Marek's disease  
viruses (1, 2 and 3 (HVT) serotypes) or the Gumboro  
disease virus. These viruses can therefore be produced  
on this cell line.

20

### **EXAMPLE 4: Multiplication of canarypox on TCF-4.10 cells.**

The TCF-4.10 cells are seeded in a roller  
bottle. The canarypox is inoculated onto an established  
25 lawn. When the cytopathic effect engendered by the  
virus has become generalized, harvesting is carried out  
by shaking so as to detach the cell lawn. The harvested  
mixture therefore consists of the cell lawn and the  
culture supernatant. The whole is homogenized by  
30 treating with an Ultraturrax for 1 min at 13,500 rpm  
(T25-type IKA appliance).

The infectious viral titre is determined by  
means of a micromethod carried out on a 96-well plate.  
The virus dilutions are inoculated onto a lawn prepared  
35 from secondary chick embryo cells. Each viral dilution  
is inoculated onto 6 wells. The plates are incubated in  
a CO<sub>2</sub> incubator for 8 days. The presence of the virus  
in the wells is checked by observing the characteristic  
cytopathic effect (CPE) under the microscope. The

infectious titre is calculated by the KARBBER method and is expressed by the logarithm of the inverse of the viral dilution which gives 50% CPE [titre =  $d+r/Nx(n+N/2)$ ], where d is the dilution expressed in  
5 logs when all the wells are positive, r is the dilution ratio, N is the number of wells per dilution and n is the number of positive wells between 0 and 100%.

Results: The viral titres obtained are equivalent to those obtained on primary duck embryo cells.

10

**EXAMPLE 5: Integration of the bcl-2 gene**

A vector which permits expression of the bcl-2 gene under the control of the CMV (human cytomegalovirus) promoter is transfected into the TDF-2A and  
15 TCF-4.10 cells using conventional transfection methods (DMSO method described by Kawai and Nishizawa (1984), Mol. Cell. Biol. 4: 1172-1174 or lipofectamine method in accordance with the supplier's (GIBCO-BRL) recommendations).

20 After the transfected cells have been selected, expression of the Bcl-2 protein is detected by Western blotting.

The cells which express the Bcl-2 protein are then tested for their ability to survive under culture  
25 conditions in which an apoptosis process is observed (maintenance of the cells at confluence).

Thus, in the case of the TDF-2A bcl-2 cells, the apoptosis process engendered by the cells arriving at confluence is deferred by from 3 to 4 days as  
30 compared with the TDF-2A cells. An increase in cell density at confluence is observed in the TCF-4.10 bcl-2 cells as compared with the TCF-4.10 cells.

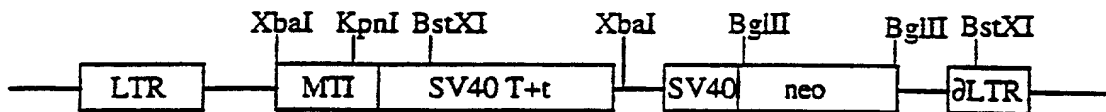
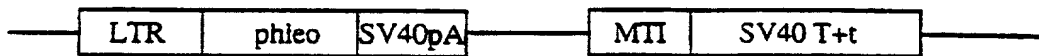
## CLAIMS

1. Avian cell line, which is immortalized, but untransformed, and resistant to apoptosis.
- 5 2. Avian cell line according to Claim 1, characterized in that it is obtained from cells of avian tissues.
3. Avian cell line according to Claim 2, characterized in that it is derived from fibroblasts or  
10 epithelial cells.
4. Immortal, untransformed avian cell line, which is selected from the group consisting of:
  - cell line TDF-2A bcl-2, which is deposited in the CNCM (Collection Nationale de Cultures de  
15 Microorganismes de l'Institut Pasteur [Pasteur Institute National Collection of Microorganism Cultures]) under reference number I-1709.
  - cell line TCF-4.10, which is deposited in the CNCM under reference number I-1710
  - 20 - cell line TCF-4.10 bcl-2, which is deposited in the CNCM under reference number I-1711.
5. Immortal avian cells which are derived from the cell line according to one of Claims 1 to 4.
6. Cells according to Claim 5, characterized in  
25 that they contain at least one expression cassette which comprises at least one nucleotide sequence encoding a molecule of industrial relevance.
7. Cells according to Claim 6, characterized in that the nucleotide sequence encodes a viral subunit of  
30 the peptide, protein or glycoprotein type or encodes protein molecules such as hormones.
8. Cells according to Claim 5, characterized in that they are infected, preferably chronically, with a virus which is able to multiply in these cells.
- 35 9. Cells according to any one of Claims 5 to 8, characterized in that they contain a survival or anti-apoptotic gene other than bcl-2, which gene is preferably selected from the group consisting of p19E1B from human adenovirus, LMP-1 from Epstein Barr virus,



BHRF1 from Epstein Barr virus, ICP34.5 from herpes simplex virus and p35 from baculovirus.

- 5      **10.**      Cells according to any one of claims 5 to 9, characterized in that they integrate vectors which are able to overexpress one or more of the genes involved in controlling the cell cycle in order to increase the rate of proliferation.
- 10      **11.**      Cells according to any one of Claims 5 to 10, characterized in that they integrate genes which encode viral receptors.
- 15      **12.**      Cells according to any one of Claims 5 to 11, characterized in that they integrate oncogenes which are able to accelerate cell growth.
- 15      **13.**      Method for producing molecules of industrial relevance or viruses, comprising culturing cells according to any one of Claims 5 to 12.

FIG. 1FIG. 2

# DECLARATION FOR USA PATENT APPLICATION

(including Design and National Stage PCT)

Attorney's Docket ID: 1

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below adjacent to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention

entitled "Immortal avian cells".

\_\_\_\_\_, the specification of which

(check one)    is attached hereto.

X is U.S. Application Serial No. 194,025 which was filed on November 19, 1998

and (if applicable) amended on \_\_\_\_\_.

   is International Application No. \_\_\_\_\_ which was filed on \_\_\_\_\_.

and (if applicable) amended on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s) (   ADDITIONAL APPLICATIONS IDENTIFIED ON ATTACHED SHEET):

Number	Country	Day/Month/Year Filed	Priority Claimed Yes No
<u>96 06 630</u>	<u>FRANCE</u>	<u>23 May 1996</u>	<u>X</u>

hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below; and insofar as the subject matter of each claim of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application in accordance with 37 C.F.R. 1.63(d). (   ADDITIONAL APPLICATIONS IDENTIFIED ON ATTACHED SHEET.)

Application Serial No.	Day/Month/Year Filed	Status -- patented, pending, abandoned
<u>PCT/FR97/0897</u>	<u>May 22, 1997</u>	<u>Pending</u>

hereby appoint the following to prosecute and transact all business in the Patent and Trademark Office connected therewith:

<u>Andrew E. Taylor-17000</u>	<u>Thomas P. Sarro-19396</u>	<u>Harold L. Novick-26011</u>	<u>Mark J. Gutttag-33057</u>
<u>Walter C. Gillis-22086</u>	<u>Ross F. Hunt, Jr.-24082</u>	<u>Douglas E. Jackson-28518</u>	<u>Daniel C. Mallery-33532</u>
<u>Marvin Petry-22752</u>	<u>William E. Jackson-24016</u>	<u>B. Aaron Schulman-31877</u>	

Direct all telephone calls to \_\_\_\_\_ TEL (703) 920-7200.

Address all correspondence to LARSON & TAYLOR, 727 23rd Street South, Arlington, VA 22202; Facsimile Tel: (703) 892-8428.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor	<u>BOUQUET Jean-Francois</u>	Citizenship	<u>FRENCH</u>
Post Office Address	<u>40 chemin de l'hopital 69280 St CONSOLIDE FRANCE</u>		
Residence - City, State (if different from P.O. address)	<u>SAME AS ABOVE FRX</u>		
SIGN AND DATE HERE	Inventor's Signature: <u>[Signature]</u>	Date:	<u>1.02.99</u>
Full Name of Second Joint Inventor, if any	<u>CLEUZIA Catherine</u>	Citizenship	<u>FRENCH</u>
Post Office Address	<u>16 Rue de l'Espérance - 69003 LYON, FRANCE</u>		
Residence - City, State (if different from P.O. address)	<u>SAME AS ABOVE FRX</u>		
SIGN AND DATE HERE	Inventor's Signature: <u>[Signature]</u>	Date:	<u>1.02.99</u>
Full Name of Third Joint Inventor, if any	<u>SAMARUT Jacques</u>	Citizenship	<u>French</u>
Post Office Address	<u>169 bis Route de Genas 69100 VILLEURBANNE France FRX</u>		
Residence - City, State (if different from P.O. address)	<u>169 bis route de Genas 69100 Villeurbanne France</u>		
SIGN AND DATE HERE	Inventor's Signature: <u>[Signature]</u>	Date:	<u>1.02.99</u>

SEE ATTACHED SHEET FOR SIMILAR INFORMATION AND SIGNATURE FOR ADDITIONAL JOINT INVENTORS.  
Law Offices of LARSON & TAYLOR, 727 23rd Street South, Arlington, Virginia 22202

400 Date 1.02.99 Third Inventor DESPETRE Phil 27  
Le Treuil - 35, chemin First Name Middle Initial Last Name  
de la Vernique  
Residence 69130 ECULLY France Signature Le Treuil - 35, chemin de la Vernique  
FRX Post Office Address 69130 ECULLY France  
Citizenship French

Date \_\_\_\_\_ Fourth Inventor \_\_\_\_\_  
First Name Middle Initial Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_

Date \_\_\_\_\_ Fifth Inventor \_\_\_\_\_  
First Name Middle Initial Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_

Date \_\_\_\_\_ Sixth Inventor \_\_\_\_\_  
First Name Middle Initial Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_

Date \_\_\_\_\_ Seventh Inventor \_\_\_\_\_  
First Name Middle Initial Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_

Date \_\_\_\_\_ Eighth Inventor \_\_\_\_\_  
First Name Middle Initial Last Name  
Residence \_\_\_\_\_ Signature \_\_\_\_\_  
Post Office Address \_\_\_\_\_  
Citizenship \_\_\_\_\_